Journal of Experimental Botany, Vol. 56, No. 421, pp. 2885–2895, November 2005
doi:10.1093/jxb/eri283 Advance Access publication 10 October, 2005
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Journal of Experimental Botany

RESEARCH PAPER

Utilization of tomato microarrays for comparative gene expression analysis in the Solanaceae

Shanna Moore[1,*,†], Paxton Payton[2,*,‡], Mark Wright[3], Steven Tanksley[3] and James Giovannoni[2,4,§]

[1] *Department of Plant Biology, Cornell University, Ithaca, NY 14853, USA*

[2] *Boyce Thompson Institute for Plant Research, Tower Road, Cornell Campus, Ithaca, NY 14853, USA*

[3] *Department of Plant Breeding and Genetics, Cornell University, Ithaca, NY 14853, USA*

[4] *USDA Plant, Soil, and Nutrition Laboratory, USDA-ARS, Tower Road, Cornell Campus, Ithaca, NY 14853, USA*

Received 28 March 2005; Accepted 5 August 2005

Abstract

Transcriptional profiling allows for the assessment and comparison of cross-species gene activity and function on a comprehensive scale. The Solanaceae is a large, diverse dicot family, with well-established genetic relationships between major crop species (tomato, potato, pepper, eggplant, and tobacco). Although *Arabidopsis thaliana* is often the model of choice for anchoring comparative studies, certain biological processes are better examined in other plants. The ripening of fleshy fruits is not tractable in *Arabidopsis*; however, it has received considerable attention in tomato. As a member of the Solanaceae, tomato provides a well-characterized system to anchor transcriptional profiles of fruit ripening and development in related species. By utilizing different stages of tomato, pepper, and eggplant fruit, the use of tomato microarrays for expression analysis has been demonstrated in closely related heterologous species, and groups of candidate expressed sequence tags, which are useful as orthologous markers, have been identified, as well as genes implicated in fruit ripening and development in the Solanaceae.

Key words: Eggplant, fruit ripening microarray, pepper, Solanaceae, tomato.

Introduction

The development of cost-effective high-throughput sequencing technologies has resulted in the accumulation of valuable genome sequence information from an assortment of 'model' organisms including *Escherichia coli*, yeast, human, *Drosophila*, *C. elegans*, *Arabidopsis thaliana*, and rice (*Oryza sativa*). Full sequence information, coupled with a growing number of extensive expressed sequence tag (EST) projects, now allows for inter-species sequence comparisons on the micro- and macro-levels, revealing insights into genome structure in addition to evolution and divergence mechanisms (Nadeau and Sankoff, 1998; Heslop-Harrison, 2000; Mitchell-Olds and Clauss, 2002; Schmidt, 2002). Cross-species genome comparisons and the resulting information concerning sequence level co-linearity are valuable tools providing a means for gene discovery and analysis of evolution and crop domestication. In the plant community, extensive sequence information is currently only available for *Arabidopsis* and rice (Arabidopsis Genome Initiative, 2000; Yu *et al*., 2002), although international efforts have recently been initiated for *Medicago*, lotus, and tomato. It is therefore imperative to investigate the degree to which available sequence resources can be exploited for discovery in species for which less information and fewer resources are currently available. To date, relationships, at the genome sequence level, have been demonstrated within the Poaceae, Solanaceae, Brassicacea, Fabaceae, and pines, as well as between families (e.g. *Arabidopsis*/rice, *Arabidopsis*/tomato, *Arabidopsis*/moss) (Lagercrantz and Lydiate, 1996; Livingstone *et al*., 1999; Ku *et al*., 2000; Thorup *et al*., 2000; Fulton *et al*., 2002; Salse *et al*., 2002; Bennetzen and Ma, 2003; Izawa *et al*., 2003; Nishiyama *et al*., 2003). These studies have revealed that, although

* These authors contributed equally to this manuscript.
† Present address: Department of Plant Breeding and Genetics, Cornell University, Ithaca, NY 14853, USA.
‡ Present address: USDA Cropping Systems Research Laboratory, 3810 4th Street, Lubbock, TX 79415, USA.
§ To whom correspondence should be addressed. Fax: +1 607 254 2958. E-mail: jjg33@cornell.edu

different degrees of restructuring are observed (e.g. inversion, rearrangements), some level of gene presence and order, on the macro- and/or micro-syntenic level, can often be identified.

A primary objective in comparative sequence studies is to identify putative orthologous genes based on sequence consensus. Although studies examining and comparing linear DNA allow insight into the preservation of gene order, structure, and 'putative' functional homology, little information concerning the conservation of gene function is directly conveyed by sequence analysis alone. The behaviour of single genes, or specific gene families, is often examined in depth across diverse genotypes, yet there are few studies conducted in such a way as to allow for the assessment of the conservation of cross-species gene function on a larger scale. The development of EST and microarray technology makes it possible to incorporate transcriptional information into comparative genomics studies; however, this resource remains largely unexploited with few examples to date (Horvath *et al*., 2003; Fei *et al*., 2004).

Tomato is a member of the Solanaceae and has long served as the model system for examining climacteric fruit ripening, resulting in the accumulation of many resources including an extensive germplasm collection, numerous mutants, a dense molecular map, comprehensive EST dataset (http://www.sgn.cornell.edu/), and a recently available cDNA microarray (reviewed in Adams-Phillips, 2004; Giovannoni, 2004). These resources have facilitated comparisons within the Solanaceae and resulted in extensive cross-species studies that rival those present among the grasses (Livingstone *et al*., 1999). The Solanaceae is a large (>3000 species), extremely diverse family containing species with origins in both the Old (eggplant – China, India) and New World (pepper/potato/tomato – Central and South America) (Knapp, 2002). The family has both economic and nutritional value. Humans utilize >18 species in this family, and those that are consumed in the form of vegetable crops provide significant dietary sources of vitamins A and C and antioxidants (e.g. lycopene) (Davies *et al*., 1991; Canfield *et al*., 1993; Bramley, 2000). Many species in the Solanaceae have the same basic chromosome number (x=12), and the genic content of tomato, potato, pepper, and eggplant remains remarkably similar despite differences in genome size (~950 Mb, 1800 Mb, 3000 Mb, and 1100 Mb, respectively) and varying numbers of chromosomal rearrangements (Bonierbale, 1988; Livingstone *et al*., 1999; Doganlar *et al*., 2002*a*). The genomes of tomato and potato differ by only five paracentric inversions; while those of pepper and tomato and eggplant and tomato show conservation of linkage blocks, yet exhibit more extensive rearrangements. Although it is clear that conservation of both genic content and gene position exist between key members of the Solanaceae, a vast range of phenotypes is present within the family. Nevertheless, there is evidence of conservation of quantitative trait loci (QTLs) relating to domestication traits among tomato, pepper, and eggplant (Gephardt *et al*., 1991; Tanksley *et al*., 1992; Thorup, 2000; Doganlar *et al*., 2002*b*). This phenotypic diversity is thus likely to be the result of differential transcriptional regulation of similar gene sequences. In a compilation of results from multiple QTL studies of fruit quality traits (e.g. weight, shape, colour), relatively few loci are implicated overall in the drastic phenotypic changes observed in the domestication of fruit from wild relatives in tomato, pepper, and eggplant (the three major fruit consumed from the Solanaceae) (Doganlar *et al*., 2002*b*; Frary *et al*., 2003; van der Knapp and Tanksley, 2003). Additionally, two major QTL in tomato, *fw2.2* (fruit weight) and *ovate* (shape) have also been implicated in eggplant and pepper (Frary *et al*., 2000; van der Knapp and Tanksley, 2003).

With the clear demonstration of substantial gene conservation and the public availability of a tomato cDNA microarray (Alba *et al*., 2004), the Solanaceae provide an excellent foundation to examine transcriptional profiles of fruit ripening and development in three related, yet phenotypically distinct, species. The data presented here will facilitate the identification of orthologous loci among three major crop species in the family, contribute evidence towards the utility of microarrays as a comparative tool, and further our understanding of transcriptional control as it relates to phenotypic diversity.

Materials and methods

Experimental design

To ensure the microarray data in this study can be interpreted and independently verified, the following parameters were used to define the array experiments based on the proposed minimum information about a microarray experiment (Brazma, 2001). All raw microarray data from this experiment can be downloaded from http://ted.bti.cornell.edu/cgi-bin/miame/home.cgi for public analysis and scrutiny.

A direct comparison, dye-swap design was employed to monitor gene expression changes between immature and mature fruit tissues and differences between tomato (*Solanum lycopersicum* cv. 'Ailsa Craig'; note: tomato has recently been re-classified from *Lycopersicon esculentum*; see Olmstead *et al*., 1999; Knapp, 2002) and eggplant (*Solanum melongena* cv. 'Ichiban'), and pepper (*Capsicum annuum* cv. 'Big Dipper') (Kerr and Churchill, 2001; Kerr *et al*., 2002; Yang and Speed, 2002). For each pair-wise comparison [mature green (MG) tomato versus red ripe (RR) tomato (10 d post-breaker), immature (IE) eggplant (10 d post-anthesis; dpa) versus mature (ME) eggplant (40 dpa), mature green (MP) pepper versus red ripe (RP) pepper, RR tomato versus ME eggplant, and RR tomato versus RP pepper], a minimum of four independent RNA extracts was obtained from independently harvested, pooled tissue samples. For experiments involving mature versus immature fruit, at least four hybridizations were carried out for each species. For experiments comparing tomato and eggplant or pepper, three hybridizations were performed, with one dye-swap included in each.

Solanaceae sequence comparisons

Sequence data for tomato, eggplant, and pepper were obtained from the Solanaceae Genomics Network (SGN) in the form of FASTA files

representing the consensus sequences of SGN unigene assemblies. Unigene assemblies were designed to minimize redundant data in EST collections by aligning and assembling overlapping ESTs, which are ostensibly cloned and sequenced, from identical mRNA transcripts. The datasets used were titled '*Lycopersicon* combined Build 2', '*Solanum melongena* Build 2', and '*Capsicum* combined Build 1' for tomato, eggplant, and pepper, respectively. The datasets and detailed information on their construction are available from SGN. The *Arabidopsis* data used was the *Arabidopsis* gene model coding sequence BLAST database 'ATH1_cds' from The *Arabidopsis* Information Resource (TAIR) dated 17 April 2003. The FASTA format sequences for querying against the SGN tomato unigene were recovered using 'fastacmd' from the NCBI BLAST toolkit.

The pepper, eggplant, and *Arabidopsis* datasets were compared with the tomato dataset using NCBI BLAST version 2.2.6 in the 'BLASTn' mode. This comparison mode was used because the binding affinity dynamics of the tomato microarray is a nucleotide-to-nucleotide process and the purpose is to demonstrate the conservation of DNA coding sequence in the Solanaceae relative to that of a more distant relative (*Arabidopsis*). The tabular output (–m 8) of BLAST was collected and reduced to contain only the best hit for each query sequence. From these data, the reverse cumulative distribution of expected value scores generated by BLAST is shown in Fig. 1. The ordinate value indicated by the plotted series was interpreted as the percentage of each dataset having BLASTn matches to the tomato dataset (at or above the significance level indicated by the expected value in the abscissa).

It is noted here that the tomato, eggplant, and pepper datasets are unigene assemblies from EST data and only approximate a non-redundant collection of coding sequences. These datasets are also incomplete representations of the transcriptome, limited to the extent of sampling and the efficiency of EST sequencing. The tomato dataset is by far the largest, representing >171 516 EST sequences assembled into 30 331 unigenes, whereas the pepper dataset represents 20 722 EST sequences in 9554 unigenes, and the eggplant dataset only 3181 EST sequences in 1841 unigenes. The *Arabidopsis* dataset, however, is precisely non-redundant by design and represents the current best approximation to a complete sampling of the *Arabidopsis* transcriptome. This disparity in the quality and completeness of the datasets is not expected to change the basic relationship of substantial nucleotide conservation in the Solanaceae family demonstrated in Fig. 1. Randomized sub-sampling of the datasets established that the plotted cumulative distributions are remarkably stable (data not shown).

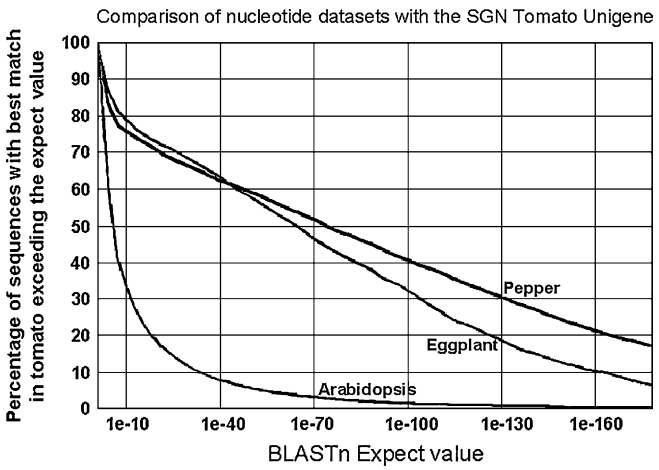

Fig. 1. Computational comparison of EST sequence data between the Solanaceae and *Arabidopsis thaliana.* EST comparisons with the tomato sequence data for pepper, eggplant, and *Arabidopsis* demonstrate substantially higher conservation at the nucleotide level amongst eggplant and pepper than that observed in the same comparison using the *Arabidopsis* coding sequence dataset from TAIR. At BLAST expect value thresholds of 1e–10, >75% of sequence data for eggplant and for pepper have a match in the tomato EST dataset, while only 35% of *Arabidopsis* coding sequences find matches. At high thresholds of 1e–70, for pepper and eggplant, 52% and 45% find tomato matches, respectively, while *Arabidopsis* drops to <5%.

Tissue collection

Tomato and pepper plants were grown from seed in the greenhouse, while eggplant seedlings were purchased commercially as seedlings and then grown in the greenhouse. All seeds and seedlings were grown in the summer (June–September) of 2003. Growing conditions were on average 28/19 °C (day/night) with high-pressure sodium supplemental lights and daily fertilization with Excell solution (200 ppm). Tomato, pepper, and eggplant flowers were tagged at anthesis and the following fruits were collected: tomato, MG (~35 dpa) and RR (10 d post-breaker); pepper, MP (~40 dpa) and RP (10 d post-breaker); and eggplant, IE (10 dpa) and ME (40 dpa). In that eggplant does not experience a marked ripening stage like tomato and pepper, immature and mature fruit were compared. Upon harvest, all fruits were acclimated for 4–8 h in the laboratory. Seeds were harvested, placental tissue was removed, and pericarp tissue was immediately frozen in liquid nitrogen and stored at −80 °C.

RNA isolation

All tissues were ground to a fine powder in liquid nitrogen and stored at −80 °C or immediately used for RNA extraction. Tomato and eggplant RNA was extracted according to the following protocol. Approximately 2 g of homogenized tissue was placed in 10 ml of 80 °C extraction buffer containing 100 mM LiCl, 100 mM TRIS-HCl (pH 8.0), 10 mM EDTA, 1% SDS, and 50% DEPC-water-saturated phenol. The extraction mixture was vortexed and, following the addition of 5 ml chloroform/isoamyl alcohol (24:1), was centrifuged at 4 °C and 11 000 *g* for 20 min. Following centrifugation, the aqueous phase was removed and RNA was precipitated with an equal volume of 4 M LiCl for 1 h at −80 °C. The precipitated RNA was pelleted by centrifugation for 20 min at 4 °C and 11 000 *g*. The precipitated pellet was washed with 70% ethanol and air-dried. The dried pellet was re-suspended in H_2O to yield a final RNA concentration of 3–7 μg μl^{-1}.

Pepper RNA was extracted according to the following protocol. Approximately 2 g of homogenized tissue were placed in 5 ml of extraction buffer containing 1.0 M TRIS-HCl (pH 9.0), 1 M NaCl, and 0.2 M EDTA (pH 9.0). Five millilitres of phenol/chloroform (1:1) were added, the mixture was vortexed, and placed on ice for 20 min. The mixture was then centrifuged at 4 °C and 5800 *g* for 20 min. Following centrifugation, the aqueous phase was removed and the nucleic acids were precipitated with 3 ml of isopropanol for 1 h at −20C. The precipitated RNA was pelleted by centrifugation for 20 min at 4 °C and 14 000 *g*. The pellet was washed with 3 ml of 70% ethanol, re-suspended in 1 ml DEPC H_2O, transferred to a new tube, and precipitated again by the addition of an equal volume of 4 M LiCl at −20 °C for 1 h. The RNA was pelleted by centrifugation for 20 min at 4 °C and 14 000 *g*. The resulting pellet was rinsed well with 3 ml of 70% EtOH and resuspended in H_2O to yield a final RNA concentration of 3–7 μg μl^{-1}.

Tomato microarray construction

Microarrays were purchased from The Center for Gene Expression at the Boyce Thompson Institute (http://bti.cornell.edu/CGEP/CGEP.html). The array contains 13 400 printed elements and ~8700 unigenes (Alba *et al*., 2004). Funding for the CGEP is partially provided by NSF#IBN-0109633.

Labelling and hybridization of fluorescent targets

The 'Array 50' 3DNA Expression Array Detection Kit from Genisphere, Inc. (Hatfield, PA, USA; http://www.genisphere.com) was utilized for cDNA synthesis and labelling. First-strand cDNA was generated from reverse transcription of 50 μg total RNA using either Cy3 or Cy5 specific primers. Hybridizations were performed according to the two-step protocol shown in Appendix B of the manufacturer's handbook.

Image acquisition

Image acquisition (utilizing 10 μm resolution) was performed using a ScanArray 5000 (Packard BioScience, Meriden, CT, USA). In general, laser power was set at 85% with a PMT gain setting of 75%, although minor changes in laser power and gain were used based on signal intensities for each individual hybridization. All hybridizations were done in pair-wise fashion and reciprocal (dye-swap) hybridizations were performed to replicate data. For the tomato ripening profile, four hybridizations (two dye-swap sets) were performed for each comparison, and three hybridizations were done (with one dye-swap set) for pepper and eggplant.

Image analysis

Signal intensities were quantified using ImaGene 5.5 software (BioDiscovery, Los Angeles, CA, USA). Expression signal and background signal segmentation were achieved by omitting the top and bottom 10% of the pixel values for each spot prior to quantification. Visually flagged and low quality spots (less than two times the background corrected intensity divided by background standard deviation) were filtered from subsequent analysis. Normalization and analysis of microarray data were performed using GeneSpring 6.0 (SiliconGenetics, Redwood City, CA, USA). For immature versus mature comparisons, mean EST signals derived from less than three non-flagged replicate hybridization signals were defined as lacking sufficient data and removed. For comparisons between tomato and eggplant and pepper, signals derived from less than two non-flagged replicate signals were removed. Per spot and per chip normalization was achieved using the print-tip LOWESS method, with 20% of the data used to calculate the LOWESS fit at each point (Clevel and Devlin, 1988; Yang *et al.*, 2002). Significantly up- or down-regulated genes were filtered for expression ratios greater or smaller than 1.85 and 0.54, respectively, and for *t*-test *P*-value <0.05.

DNA extraction

Genomic DNA was isolated from expanding leaves of tomato, tobacco, potato, pepper (sweet bell and jalapeno), petunia, and eggplant, digested with *Dra*I and subsequently analysed by Southern blot hybridization as described previously (Tanksley *et al*., 1992).

Southern and northern blots

Ten micrograms of total RNA, for each of the following tissues: tomato cv. 'Ailsa Craig' leaf, mature green, breaker, and red ripe fruit; potato leaf, petunia leaf, tobacco leaf (*N. benthamiana* and *N. tabacum*), green pepper and red pepper fruit, and eggplant fruit, was fractionated through 1% (w/v) agarose gels containing 15% (v/v) formaldehyde. Gels were blotted onto Hybond N nylon membrane (Amersham-Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Filters were hybridized at 65 °C to ^{32}P-labelled random primed probes (Feinberg and Vogelstein, 1983), in a buffer containing 5× SSC, 0.5% (w/v) SDS, 50 mM Na-P (pH 7.5), and 5× Denhardt's solution. Hybridizations were performed for ~16 h, after which the filters were washed in 2× SSC, 0.1% (w/v) SDS and then 1× SSC, 0.1% (w/v) SDS at 65°C. Signal intensity was visualized by autoradiography using XAR-5 film (Kodak, Rochester, NY, USA) with two intensifying screens at −80 °C.

Results and discussion

Experimental design

A functional genomics approach, employing cDNA microarrays, was used to compare transcriptional differences in ripened and non-ripened fruit from the three major fruit-bearing Solanaceous species: tomato, pepper, and eggplant. Three sets of microarray hybridizations were performed for this study, with each set representing a comparison between two distinct stages of fruit development: (i) mature green (MG) and red ripe (RR) tomato fruit; (ii) mature green (MP) and red ripe (RP) bell pepper fruit; (iii) immature (IE) and mature (ME) eggplant fruit (see Materials and methods for detailed explanations of stages).

Sequence comparisons

The Solanaceae Genomics Network (SGN; http://sgn.cornell.edu) provides a comprehensive collection of Solanaceae-related information and tools to the public. There are currently 'unigene' assemblies available on SGN for tomato, potato, pepper, and eggplant, with an 'assembly' defined as a minimally redundant collection of alignments derived from ESTs ostensibly from the same putative transcript (detailed information on assembly procedures may be found at www.sgn.cornell.edu). To support the notion of the tomato cDNA arrays as a viable tool for gene discovery in other solanaceous species, an *in silico* comparison of the current unigene builds of tomato, pepper, and eggplant was performed (Fig. 1). Potato was not included as the focus of this study was on fruit development and ripening. At BLAST threshold values of 1e–10, >75% of the available sequence for pepper and eggplant has a match in tomato. A similar comparison of *Arabidopsis* and tomato resulted in only 35% of available sequences finding matches. At a higher threshold of 1e–70, 52% of pepper sequences and 45% of eggplant sequences find tomato matches, while *Arabidopsis* drops to <5% (see Materials and methods for details). These results indicate a substantial level of nucleotide conservation among available sequences for tomato, pepper, and eggplant, and support the utilization of the tomato cDNA array for gene discovery in other species of the Solanaceae. An even higher level of nucleotide conservation may ultimately be observed as more sequences become available for all the species considered.

Microarray utility for comparative gene expression analysis

When heterologous target cDNAs were created from pepper and eggplant and applied to the tomato array, the overall quality of hybridization observed was consistent with that seen for tomato hybridizations. Table 1 shows that ~8200 ESTs (61% of the total 13 440 elements on the array) yielded detectable signal when hybridized with a

Table 1. *Average number of ESTs detected in solanaceous fruit hybridizations to the tomato cDNA microarray (Tom1)*

The number of ESTs detected represents the average number in at least four hybridizations that passed all signal criteria (see Materials and methods). Differentially expressed ESTs represent those showing at least 2-fold changes in expression at a *t*-test *P*-value <0.05 and *n* >3. MG, mature green; B+10, breaker + 10 d (red ripe); GP, mature green pepper; RP, red ripe pepper; YE, 10 d post-anthesis (immature fruit); OE, 40 d post-anthesis (mature fruit).

Tissue	Average no. of genes detected	Differentially expressed
Tomato fruit	8197±434	1051 (MG) 939 (B+10)
Pepper fruit	6120±598	723 (GP) 635 (RP)
Eggplant fruit	6934±254	783 (YE) 697 (OE)

tomato cDNA pool. These ESTs represent 5408 unigenes. Approximately 52% (>7000 ESTs, 4115 unigenes) and 45% (>6000 ESTs, 3100 unigenes) of the tomato ESTs showed consistently detectable expression levels in eggplant and pepper tissues, respectively.

Reliable molecular clock datas are not available for the Solanaceae, making it difficult to assign a more precise distance between any of the species considered. Based on phylogenetic data, it is expected that a greater number of genes would exhibit homology between tomato and eggplant. Both species belong to the genus *Solanum* (Olmstead *et al*., 1999) whereas pepper is classified within the *Capsicum*. Although all three belong to the same tribe of Solanai, *Capsicum* is considerably distant from *Solanum*.

These results also clearly show that tomato arrays are viable tools for analysis of pepper and eggplant gene expression. When considering the distribution of ESTs expressed among the hybridizations as shown in Fig. 2, 28% of the elements on the array (2023 unigenes) are expressed in all three species. This set is significant in that it reflects loci common to solanaceous fruit and provides a large pool of prospective markers for comparative studies (Supplementary data Table 8; http://ted.bti.cornell.edu/cgi-bin/miame/home.cgi: follow the 'Solanaceae fruit comparison' link to the 'download' link to retrieve all supplemental data mentioned in this manuscript). As predicted, based on established phylogeny, more expressed ESTs appear in common between tomato and eggplant than in the other comparisons. Interestingly, relative to the set of genes examined, eggplant and pepper have more expressed ESTs in common than tomato and pepper. This information may be useful in establishing a more precise order of relatedness among the three species.

Comparative analysis of transcriptional profiles in fruit of the Solanaceae

Figure 3 shows a summary of the ESTs differentially expressed for each species–species comparison and how these ESTs coincide with the other solanaceous hybridizations.

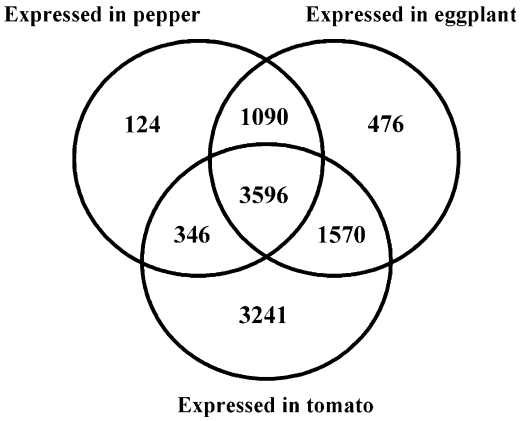

Fig. 2. Summary of expressed EST observed in solanaceous hybridizations. See Table 1 for selection criteria. 2552 ESTs were not detected in any comparison. These include sequences not expressed, expressed at low levels below the detectable range of the software utilized, marked as 'failed PCR reaction' by array quality control assessment, classified as chimeric, and those determined to contain only vector sequence.

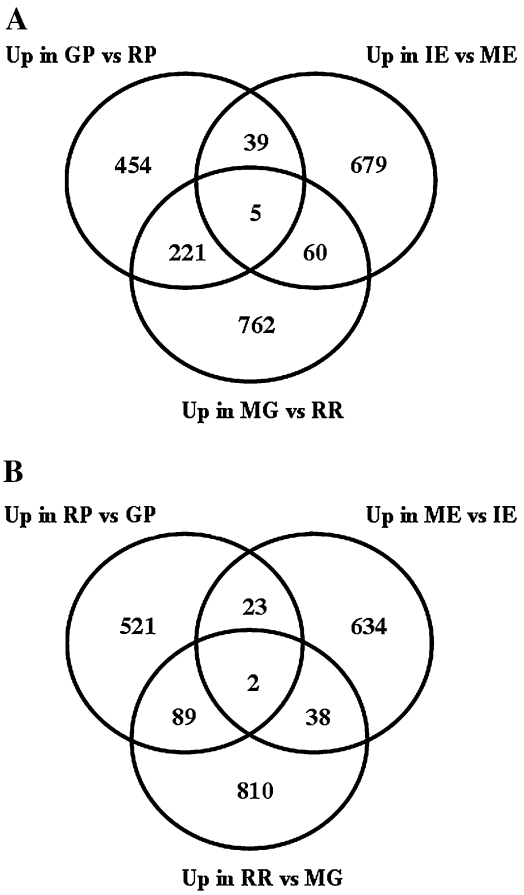

Fig. 3. ESTs differentially expressed in microarray hybridizations. (A) ESTs up-regulated immature (unripe) relative to mature (ripe) tissues; (B) ESTs up-regulated in mature relative to immature tissues. Differential expression is defined as >1.8-fold change in transcript abundance, at *t*-test *P*-value <0.05. Abbreviations: GP, green pepper; RP, red pepper; IE, immature eggplant; ME, mature eggplant; MG, mature green tomato; RR, red ripe tomato.

Of the ESTs fulfilling set quality criteria (see Materials and methods) in tomato, ~24% of the average total number detected are differentially expressed. In pepper ~22% are differentially expressed and ~21% in eggplant (a full listing of ESTs is available in Tables 2 –8 in Supplementary data). Overall, there are similar numbers of up-regulated ESTs seen in both tissues of all three species. There are, however, only five differentially expressed ESTs that are up-regulated in all immature tissues (Fig. 3A), and two ESTs commonly up-regulated in mature tissues (Fig. 3B).

This would indicate that while there are likely to be many similar processes active in the fruits for all three species (as defined by the non-variable gene expression), inducible activities might be related to the obvious variation in fruit morphologies and maturation phenotypes of the three species. Fruit from all three species are defined as berries; however, this is a broad morphological classification and marked differences are apparent. Ripe tomatoes consist of a fleshy expanded pericarp, moderately expanded placenta tissue, and solubilized gelatinous locule tissue. Ripe bell peppers have a similar fleshy pericarp (though not as expanded); however, they have minimal expansion of the placenta and hollow locules. Purple eggplant has a fleshy pericarp and a dramatically expanded placenta, giving the interior of the fruit a homogenous look and making locule determination difficult. In addition, eggplant does not undergo the same type of ripening process as pepper and tomato. As such, a considerably less mature stage of eggplant (as compared with the mature green stages of pepper and tomato) was used to maximize discovery of gene expression differences with the corresponding mature eggplant fruit.

Of the five ESTs commonly up-regulated in immature tissues, two have no known homology, one has homology to glycolate oxidase (a key enzyme in photorespiration), another matches the sequence of an inorganic diphosphatase, and the fifth is CIG 1, a B-type cyclin involved in phase transition in mitotic division (Doonan and Forbert, 1997; Barak *et al*., 2001).

Regarding the two transcripts higher in all mature tissue, one has no known homology while the other represents pectinesterase, which makes pectin more accessible to hydrolases and thus contributes to fruit cell wall modification and softening during ripening (Gray *et al*., 1992).

While there are differentially expressed ESTs in common for all of the pairwise comparisons, similarities between tomato and pepper are strongest. There are more than three times as many common ESTs up-regulated in green pepper and green tomato (in relation to their ripe counterparts) as compared with results in tomato and eggplant or pepper and eggplant. Clearly the developmental stages compared for pepper and tomato are phenotypically very similar, despite eggplant's closer phylogenetic relationship with tomato. This point is supported by the presence of the tomato cultivar 'Yellow Stuffer', in which the tomato fruit shape closely resembles a bell pepper, including a lack of locular jelly, signifying a small number of loci involved in the control of this fruit shape and size (van der Knapp and Tanksley, 2003). Although allelic mutation and domestic selection of these few loci are implicated in the resulting differences, orthologous loci have been identified in both species (and in eggplant as well) and transcriptional regulation is also likely to play a role.

There are 132 unigenes represented in the 220 common ESTs up-regulated in both mature green tomato and mature green pepper. Of these, 36% have no known homology (novel) or have homology to another protein with no known function (unknown). The next prominent class (25%) represents ESTs implicated in metabolism and energy, most notably those related to photosynthesis (e.g. chlorphyll *a*/*b* binding protein, photosystem I and II-related proteins). Smaller classes of ESTs related to DNA processing, and protein synthesis and fate are also recognized. A complete list of these ESTs is available in Table 12 in the Supplementary data.

There are fewer common transcripts in immature eggplant and MG tomato. Of the 60 ESTs (representing 34 unigenes), 40% fall into the 'unknown' or 'novel' classes. There are several transcripts related to phenylpropanoid metabolism (e.g. cinnamic acid 4-hydroxylase), which may reflect anthocyanin accumulation (Blount *et al*., 2000), in addition to several 'wound-induced' proteins and a single 'ripening-related' protein. Unlike transcripts common in MG tomato and MG pepper, there are no large recognizable classes of related transcripts.

Aside from the 'novel'/'unknown' transcripts (23%), the majority of similarly up-regulated transcripts in unripe/young pepper and eggplant hybridizations have putative homology to pathogen response-related genes (22%). Additionally, three of the 27 unigenes represented have homology to genes involved in ethylene response or signalling. Both sets of transcripts may reflect pathogen attacks on the plants during cultivation. Eggplant and pepper plants were more vulnerable (than tomato) to pests while being grown in a greenhouse for these experiments.

The phenotypic similarity also holds true for transcripts elevated in mature (ripe) fruits. Twice as many common ESTs appeared elevated in both ripe tomato and pepper (89 ESTs, 51 unigenes), as compared with tomato/eggplant or pepper/eggplant comparisons. Again the largest category represents those transcripts with novel/unknown homology (34%). ESTs that match genes with functional classification include categories of transcription factors (three unigenes), phosphatases (two unigenes), heat shock proteins (three unigenes), and transcripts implicated in cell wall modification (three unigenes). Most interesting is the transcription factor, RIN, which was recently identified in tomato and is implicated as an ethylene-independent regulator of ripening (Vrebalov *et al*., 2002) and may also participate in ripening of pepper. Ripening is physiologically divided into

two distinct classes: climacteric and non-climacteric. Climacteric ripening is distinguished from non-climacteric by an elevated rate of respiration usually accompanied by an increase in ethylene biosynthesis (Alexander and Grierson, 2002). While tomato is climacteric, both pepper and eggplant are considered to be non-climacteric, although variable patterns of ethylene evolution are often noted in these species (Rodriguez *et al*., 1999; Villavicenio *et al*., 1999; Liu *et al*., 2005).

Additionally there are multiple unigenes implicated in pathogen/stress response (e.g. NP24 and hin-1) which may reflect a response to general loss of tissue integrity during ripening. Although tomato and pepper both undergo a dramatic ripening programme, many end-products related to colour, flavour, and taste may be different.

As mentioned, tomato is climacteric and relies heavily on the action of ethylene to ripen (Yen *et al*., 1995; reviewed in Giovannoni, 2004), whereas pepper does not seem to require ethylene during ripening. Accordingly, up-regulation of over 15 unigenes related to ethylene evolution and response are observed in ripe tomato but not in ripe pepper. Additionally, a phytochrome A (PHY A)-mediated light response, independent of ethylene, has been shown to affect the ultimate magnitude of carotenoids present in ripe tomato fruit (Alba *et al*., 2000), and an increase in a PHY A signal transduction component is also seen in ripe tomato. There is little evidence for a PHY A role in pepper fruit ripening and no increase in this component was observed.

Although tomato and pepper both experience a dramatic colour change from green to red as they ripen, different compounds underlie the resulting colour. The accumulation of the carotenoid lycopene is responsible in tomato, whereas in pepper the more downstream carotenoids capsanthin and capsorubin result in the red pigmentation (Hornero-Mendez and Minguez-Mosquera, 2000; Hirschberg, 2001). An up-regulation of beta-carotene hydroxylase (which is the first step in converting beta-carotene to downstrean carotenoids) is seen only in ripe pepper. Additionally, other downstream (from lycopene) carotenoid hydroxylases are ferrodoxin-dependent and several ferrodoxin-related unigenes are seen up-regulated only in ripe pepper (Bouvier *et al*., 1998).

Direct comparisons between mature heterologous tissues in simultaneous pair-wise hybridizations were also performed. However, because all of the ESTs were derived originally from tomato, data from ESTs showing up-regulation in tomato relative to pepper or eggplant should be viewed with some caution. These results are likely to reflect differences in transcript accumulation but, in some cases, they could be due to sequence divergence and thus the inability of a related, yet divergent, transcript in pepper and eggplant to bind to the available tomato EST. This point has been addressed in Fig. 4 by hybridizing several chosen ESTs which were induced in tomato versus pepper or eggplant to genomic Southern blots representing different solanaceous species. The probes utilized hybridized to almost all species indicating the presence of homologous

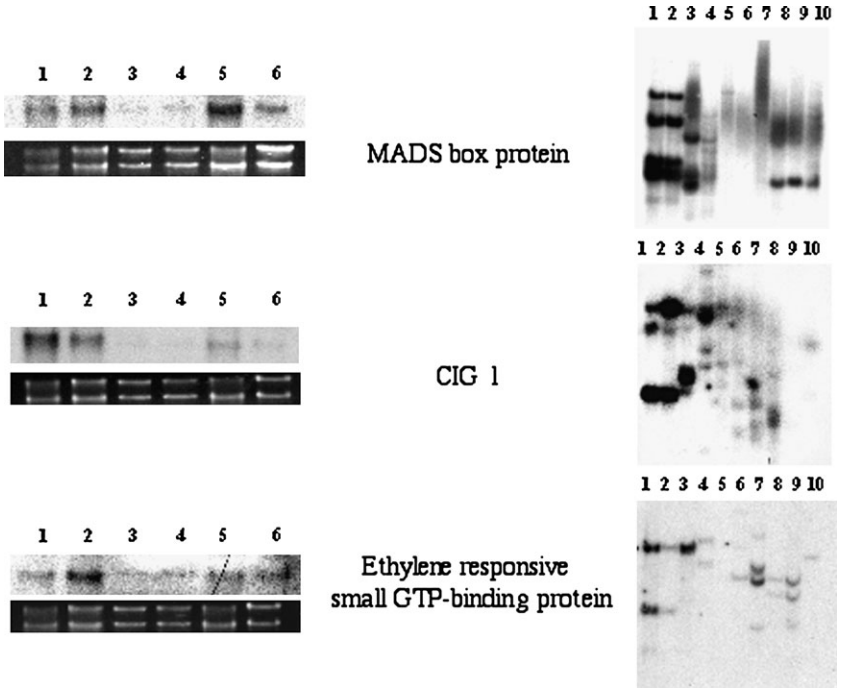

Fig. 4. Gel-blot analysis of selected clones with differential expression patterns among different solanaceous tissues. The putative functional homology assigned to the EST utilized is shown. In the northern blots, shown on the left, the tissues used are as follows: 1, mature green tomato; 2, red ripe tomato; 3, green pepper; 4, red pepper; 5, immature eggplant; 6, mature eggplant. In the corresponding Southern blots, on the right, the tissues used are as follows: 1, tomato (*Ailsa Craig*); 2, tomato (M82); 3, tomato (*S. pennellii*); 4, potato; 5, petunia; 6, tobacco (*N. benthamiana*); 7, tobacco (*N. tabacum*); 8, sweet pepper; 9, jalapeno; 10, eggplant.

sequences in the respective genomes and supporting the hypothesis that most differential expression in these comparisons is indeed due to differences in transcript accumulation. Nevertheless, the possibility that sequence divergence is the cause of apparent differential expression cannot be excluded in all cases without confirmatory experimentation.

Identification of gene expression unique to fruit of each species

In comparisons of ripe pepper to ripe tomato and mature eggplant to ripe tomato, 707 ESTs were up-regulated in ripe pepper and 1216 in mature eggplant, respectively, when compared with tomato (a full listing of ESTs is available in Tables 23 and 24 in the Supplementary data). Of these ESTs only one was unique in pepper, 706 were seen higher in both pepper and eggplant and 510 were unique in eggplant.

The common set of ESTs higher in pepper and eggplant, as compared directly with tomato, represent 384 unigenes with 34% of the ESTs having no known homology. The largest group with homology represents transcripts involved in photosynthesis and photorespiration (11%, RuBisCo, chlorophyll *a*/*b* binding protein), followed by kinases and phosphatases (5%), ribosomal genes (5%), and a small number of those implicated in protein stability and fate (2%, pepitidyl proyly *cis-trans* isomerase, heat shock proteins, ubiquitin). A gene for SYM10 also appears in this group. SYM10 is an essential factor for nodulation in legumes and is known to bind LysM motifs (Parniske and Downie, 2003). However, there is little evidence for its function in non-nodulating species.

ESTs unique to eggplant mature fruit expression include 58% with no informative homology. Smaller classes include homology to genes involved in cell wall modifications (5%, polygalacturonase and extensin), ribosomal proteins (6%), hormone response (4%, for example, related to brassinosteroids, auxin, and gibberellin), and photosynthesis (7%, for example, chlorophyll *a*/*b* binding protein, RuBisCo and subunits of PSI and PSII). While chloroplast degradation upon ripening has been demonstrated in tomato and pepper (Moser and Matile, 1997; Carrara *et al*., 2001), no reports could be found for eggplant. This is presumably due to the lack of obvious chlorophyll accumulation in maturing eggplant fruit. It would thus be interesting, based on these results, to examine the photosynthetic properties and plastid structure in ripened eggplant as there appears to be significant expression of numerous genes involved in this process.

Also of note are several unigenes with homology to vacuolar ATPases, which are involved in cellular pH regulation. There is evidence for ripening-induced increases in ATPases in tomato, as acid is an important component of fruit flavour (Coker *et al*., 2003), though little information relating to eggplant is available. It is important to note that, although similar classes of genes are represented by ESTs in common between pepper and eggplant and by those ESTs seen only in the eggplant/tomato comparison, unique unigenes appear in either set. These results may indicate common cellular mechanisms carried out by distinct genes or the ability of multiple genes, assigned the homology, to carry out similar functions.

The single EST higher in ripe pepper (in relation to ripe tomato and not seen in mature eggplant) has putative homology to enoyl CoA-hydratase/isomerase which is implicated in B-oxidation of unsaturated fatty acids (Gurvitz *et al*., 1998). This EST could be involved in unique aspects of aroma or lignification of the fruit (Allenbach and Poirier, 2000).

Expression confirmation

A subset of differentially expressed ESTs were confirmed by northern blot analysis (Fig. 4). All of the expression patterns revealed on the northern blot followed the same trends seen on the microarrays. ESTs with homology to a MADS box gene had elevated expression in immature eggplant as compared with mature eggplant and had relatively equal expression in both stages of tomato and pepper fruit considered. CIG1 was expressed higher in the microarrays in all three unripe/immature tissues as compared with their mature counterparts. Although higher expression in MG tomato and IM eggplant is seen on the northern blot, it is difficult to detect any expression in either pepper tissue. This gene may be expressed only at low levels in pepper and, while transcript differences were detectable in multiple array replicates, this northern blot may have had less sensitivity. Lastly, an ethylene-responsive GTPase binding protein was higher in ripe tomato on both the array and the gel-blot. It had similar expression in both older and younger eggplant and pepper tissues via both expression assays.

Although the current study was focused on fruit in the Solanaceae, the aim was to examine sequence conservation and ultimately lend evidence to support the utilization of the tomato arrays for additional solanaceous species. Subsequently, differentially expressed ESTs were hybridized to a series of 'salad' Southern blots including additional Solanaceae species such as tobacco, petunia, potato, and hot pepper. Selected ESTs showed varying degrees of homology to all of the other solanaceous tissues represented, suggesting tomato microarrays would be useful for gene expression and comparative genomics analysis across the broader Solanaceae (data not shown).

Summary

Microarrays utilized in a heterologous fashion can be extremely useful tools for gene discovery in species with few available resources. This utility is compounded when placed in the context of a family, like the Solanaceae, with a well-characterized genic relationship between major species (http://www.sgn.cornell.edu). Additionally, the substantial physiological and molecular information that has been

amassed with respect to tomato fruit ripening provides additional knowledge with which to correlate array data and formulate new hypotheses in relation to fruit ripening in the Solanaceae.

It has been demonstrated that the tomato cDNA array is a viable tool for gene expression profiling in pepper and eggplant and observations have been made about fruit ripening and development among the three major fruit-bearing species of the Solanaceae. The present results indicate that, while many transcripts are commonly expressed in fruit development among the species tested, several divergent mechanisms are at play, notably comparisons between tomato and pepper relative to eggplant. Given the significant difference in eggplant fruit morphology compared with tomato and pepper, this was not surprising. Markedly, the presence of transcripts involved in plastid structure and photosynthesis in eggplant suggest that the degradation of transcripts involved in these processes does not occur in eggplant as it does in tomato and pepper. While the ripening expression profiles of tomato and pepper share more similarities compared with eggplant, significant differences were detected. Given that tomato exhibits climacteric ripening, while pepper does not, transcripts involved in ethylene signalling and climacteric ripening processes were more prevalent in tomato. For pepper, ESTs with homology to lipid oxidases and carotenoids downstream in the pathway from lycopene were identified. It should be noted here that the microarray utilized in this study represents only a fraction of the genes in the tomato genome (~25% based on the model presented in Van der Hoeven *et al*., 2002). The availability of a larger and more comprehensive array representative of a larger portion of the tomato/Solanaceae gene space would certainly lead to additional discoveries.

Given the wealth of mapping and comparative data available within the Solanaceae, it is not unexpected that the tomato cDNA array can be utilized for other closely related species. What was unforeseen, however, is the extent to which these experiments were successful and they have begun to reveal new relationships regarding fruit development and ripening. The utility of the tomato array as a tool for expression profiling in the Solanaceae has been established. With the resulting data, it is possible to build a comparative transcriptional picture of the processes related to fruit development and ripening that may be integrated into current knowledge, contributing to the understanding of evolution and divergence mechanisms of agronomically important crop plants.

Acknowledgements

The authors would like to thank the laboratories of Maureen Hanson, Dan Klessig, Greg Martin, and Joyce Van Eck for providing access to petunia, tobacco (Klessig and Martin), and potato tissue, respectively. We are grateful to Dol Choi and Sanghyeob Lee for access to pepper EST sequences prior to publication. Special thanks to Ryan McQuinn for assistance with the graphics depicted in this publication. This work was supported by grants from the National Science Foundation (DBI-0116076, DBI-0211875, and DBI-0501778), the Initiative for Future Agriculture and Food Systems (2001-52100-11347) and USDA, NRI, CREES 2004-35300-14596.

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